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13. ABSTRACT ***(Maximum 200 Words)***

Introduction: Lymphangiogenesis occurs in parallel with maturation of the blood vascular system, aberrant lymphangiogenesis contributes to interstitial protein accumulation. Lymphedema therefore, ensues as a result of lymphatic blockage, trauma or dysfunction. Objective: To promote Lymphangiogenesis by stimulating lymphatic endothelial cell (LEC) proliferation and migration using an Alginate-VEGF-C hydrogel *in vitro*. Methods: Sterile alginate in solution was added to sterile VEGF-C at concentrations of 50, 100 and 200 ng/ml. The alginate-VEGF-C mixture was solidified under sterile conditions by internal geleation method (Ca^{2+}). LEC were placed on a dish and cultured with the various alginate-VEGF-C hydrogels. Results: The LEC migrated towards all of the alginate-VEGF-C hydrogels and proliferated along their length. However, the higher concentration of 200 ng/ml of VEGF-C exhibited a more profound effect on migration as well as proliferation, forming tubular structures. Plane alginate hydrogel had no effect on LEC migration or proliferation. Conclusions: The results of this *in- vitro* study indicate that an alginate biodegradable hydrogel may be an effective delivery system for VEGF-C. Its potent mitogenic effect on LEC could play an important role in Lymphangiogenesis. Further in vivo animal studies are required to evaluate alginate-VEGF-C hydrogels and their potential role in restoring the lymphatic circulation in Lymphedema.

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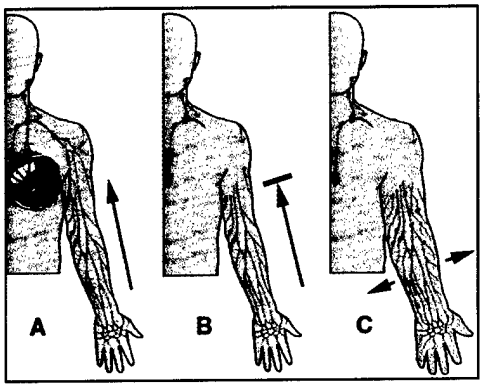
INTRODUCTION:

Breast carcinoma continues to be the most frequently occurring carcinoma in women. Presently, approximately one in every eight women will develop the disease.[1] Great strides have been made in the treatment of breast carcinoma that have reduced the risk of recurrence and improved survival rates.

The removal of axillary lymph nodes has been an integral part of breast carcinoma treatment since the end of the last century.[2] Axillary lymph nodes are removed to provide accurate information for staging, to accomplish local control, and for prognosis in order to plan adjunctive, systemic therapy. The status of the axillary lymph nodes remains the single most important predictor of survival.[3,4] Therefore, axillary treatment surgically and/or with radiotherapy is still an essential component in the management of most patients with invasive breast carcinoma.[5,6]

Chronic edema of the arm, commonly called Lymphedema or post mastectomy edema (PME), was described first as a side effect of mastectomy operations by Halsted in 1921[7] *(Figure 1)*. Although there has been a trend toward more conservative surgery and greater use of radiotherapy, PME remains a common iatrogenic problem.



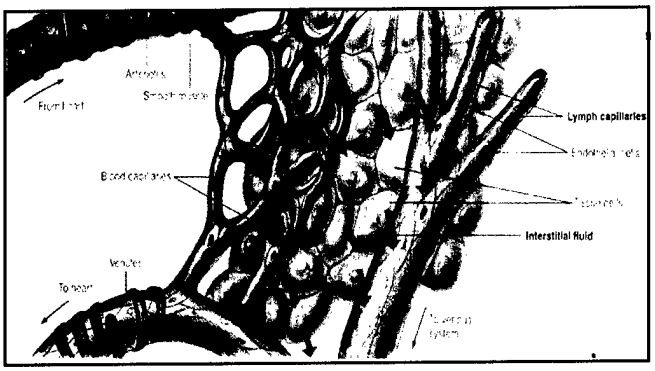
Edema represents an increase in interstitial fluid volume sufficient to manifest with swelling. Any edema, whatever the underlying cause, is due to an imbalance between capillary filtration and lymph drainage.[8] Most examples of limb edema are caused by an increase in capillary filtration, overwhelming lymph drainage capacity. Lymphedema, however, strictly occurs when swelling is due to a failure of lymph drainage in circumstances in which capillary filtration is not increased.

Figure 1: Schematic representation of Secondary Lymphedema. A) Normal lymphatic flow from the hand to the Thoracic duct (arrow). B) Removal of cancerous breast and axillary lymph nodes, lymphatic flow interrupted (arrow). C) Lymph (proteins) stagnation within the arm (arrows) and subsequent swelling.

The lymphatic system is a one-way drainage route designed to rid the "tissues" of unwanted material and excess fluid. It therefore represents a waste route and overflow pipe, with its essential function being to return to the blood vascular compartment protein, colloids, and particulate matter too large to reenter the blood compartment directly [9] *(Figure 2)*. Two types of lymphatic vessels exist: First, the smaller initial lymphatic, which includes the smallest lymphatic capillary and the larger precollector vessel, and second, the collecting lymphatic vessel into which the precollectors drain.[10]

The collecting lymphatics are the main limb lymphatic vessels that provide the afferent flow to the lymph nodes. They behave like a series smooth muscle "hearts" that are responsible mainly for the propulsion of lymph centripetally.[11] Intrinsic pumping of collecting vessels is the essential motor for lymph propulsion.

For initial lymphatics, however, flow of interstitial fluid and macromolecules is caused by intermittent changes in hydrostatic and oncotic pressures locally. [12]



Deformation or movement of the tissues by surface pressure or underlying muscle contractions and by other contractile structures, such as arterioles, causes expansion or compression of the of the initial lymphatics. The compression forces lymph along initial lymphatics. Valves in the initial and collecting lymphatics ensure that flow is unidirectional.

Figure 2. Schematic representation of Lymphatic Vessel, Arteriole and Venule.

Lymphedema arises when an intrinsic fault develops within the lymph-conducting pathways (primary lymphedema) or when damage occurs from one or more factors originating outside the lymphatic system, such as surgical removal of lymph nodes (secondary lymphedema). Lymphedema therefore, is a disease characterized by abnormal collections of fluid and proteins within the interstitial space. Treatment options for this debilitating condition have included drug therapy, physical therapy, and surgical approaches that have yielded limited success. Unfortunately, for lymphedema today, treatment options are all palliative, for there is **no effective treatment** that could offer a permanent cure to this disease.

Secondary Lymphedema resulting after iatrogenic, surgical disruption of the Lymphatic vessels in breast cancer surgery, has continued to be neglected in the U.S. in spite of the fact that it has now become an acceptable diagnosis (ICD9-457.0)[13]. Swelling of the upper limb, constitutes the most invalidating complication of breast carcinoma treatment.[14] Untreated, upper extremity lymphedema predisposes women to the development of severe acute or chronic infections with limitations on functioning and serious disturbances in a patient's quality of life. Psychological distress (disfigurement), depression and social inhibition.[15]

It is estimated that 15-20% of the 2 million breast carcinoma survivors, are living currently with post-treatment lymphedema .[16-17] The swollen arm, which can be as much as twice the normal size, is disfiguring and commonly causes functional impairment, psychosocial maladjustment and psychological morbidity.[18] Added to the physical symptoms that patients must cope with, is the pain caused unintentionally by clinicians that, interested in carcinoma recurrence, trivialize the no lethal nature of lymphedema.

BODY:

Tissue engineering is an interdisciplinary field that incorporates principles of engineering and polymer chemistry into the biological sciences, in efforts to develop biological substitutes for failed tissues and organs. It is a new and rapidly expanding field, in which the techniques are being developed for culturing or promoting regeneration of a variety of tissues, both in vitro and in vivo using polymer "scaffolds" to support tissue growth. Polymer scaffolds used in tissue engineering are generally biodegradable, often involving compounds which are already approved for human implantation. In some cases, these polymers may be chemically modified to exhibit selective cell adhesion properties, which enhance cell attachment and subsequent tissue growth.[19]

Unlike the solid polymer systems currently used to create a cell-polymer construct, a liquid support matrix that polymerizes to a gel would have the potential for injectable delivery, which would be much less invasive than open implantation. For all these reasons, calcium **alginate gels were investigated as a means of delivering Vascular Endothelial Cell Growth Factor–C (VEGF-C) to promote Lymphatic endothelial cell proliferation & migration in an *in vitro* model.**

Alginate hydrogels have been increasingly important in biotechnology applications, such as tissue engineering, artificial organs and drug carriers, specially for proteins.[20] Delivery rate can be predetermined and its local, rather than systemic administration be of great advantage.

Alginate Gels:

Alginates are approved by several regulatory authorities, such as the Food and Drug Administration, for human use as wound dressing material and as food additives.

Alginate refers to a family of polyanionic copolymers derived from brown sea algae and comprising 1,4-linked β-D-manuronic (M) and α-L-glucoronic (G) acid residues in varying proportions. The fact that G and M are C5 epimers results in a switch-over of the monomer chair conformation, giving rise to all four possible glycosidic linkages and at the molecular level, large effects like cavity formations between G residues are observed. It has been shown that the occurrence of G and M within the alginate molecule is block-wise and not random.[21]

Alginate is soluble in aqueous solutions, at room temperature, and forms stable gels in the presence of certain divalent cations, such as calcium, barium and strontium. The unique properties of alginate, combined with its biocompatibility, hydrophilicity and relatively low cost, have made it an important polymer in pharmaceutical applications.[22]

There is a direct dependence between the mechanical strength of an alginate gel and the porosity of the gel network. When gels are made from alginate rich in glucoronic (G) acid residues, higher moduli are obtained compared to gels made from alginates less enriched in G residues, and also higher diffusion rates. The proposed explanation for this behavior and

their short elastic segments become a more stiff open static network compared to the more dynamic and entangled network structure of the low-G gels with their relative long elastic segments *(Figure 3)*.

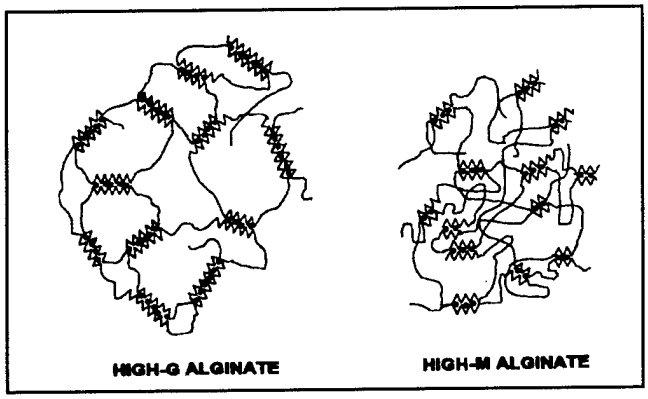


Figure 3. Schematic model for network structure in gels made from alginates with glucuronic acid blocks of different lengths.

Gelling properties of alginate are a function of the M/G composition and the sequential structure of M and G along the alginate chain. In general terms, an increase in G content (measured as $G_{n>1}$) as well as in molecular weight will give a stronger gel.

In the solid phase, the technological properties of the final product are largely governed by alginate variables such as chemical composition and sequence and molecular weight distribution. Important technological properties are gel strength, porosity/diffusion, alginate distribution, swelling/shrinking,transparency,leaching of alginate from the gels.

Because of all the different characteristics previously mentioned we decided to produce an alginate gel that would have the best properties of both, the G and M copolymers. Thus providing a gel that would be highly porous and relatively strong.

In contrast to most gelling polysaccharides, alginate gels have the particular feature of being "cold setting"'. This implies that the setting of alginate gel is more-or-less independent of temperature.

Essentially there are two main methods for the preparation of alginate gels: The dialysis/diffusion method and internal geleation method. In the dialysis/diffusion method (diffusion setting) gelling ions are allowed to diffuse into the alginate solution. This method is most commonly used in biotechnology when making alginate gel beads for immobilization purposes and also in food applications by generation of restructures foods.

An alginate solution can, however, also be solidified by internal geleation method/internal setting, i.e. in situ gelling. By this method, a calcium salt with limited solubility, or complexed Ca^{2+} -ions are mixed with an alginate solution into which the calcium ions are released, usually by addition of slowly acting acid such as D-glucono-d-lactone (GDL). This method is chosen if the purpose is to create a homogenous, non-synertic alginate macro gel to fill the space of a given container. The main difference between internal and diffusion setting is the gelling kinetics.

In our study we incorporated sterile G (50%) and M (50%) copolymers of alginate to a solution with Potassium Phosphate and Sodium Chloride (0.1 M K_2HPO_4 and 0.135 M NaCl, pH of 7.4), which was previously sterilized by autoclave. A 1.0% Sodium Alginate solution was then mixed with 0.2 gm of $CaSO_4$ per milliliter, this solution was placed in ice prior to extrusion.

The alginate gel (AG) was then drawn with a 5cc syringe and extruded through a 25 g needle and solidified under sterile conditions by internal geleation method/internal setting. As mentioned earlier this system is based on the addition of an inactive form of the cross-linking ion (Ca^{2+}) into an alginate solution.

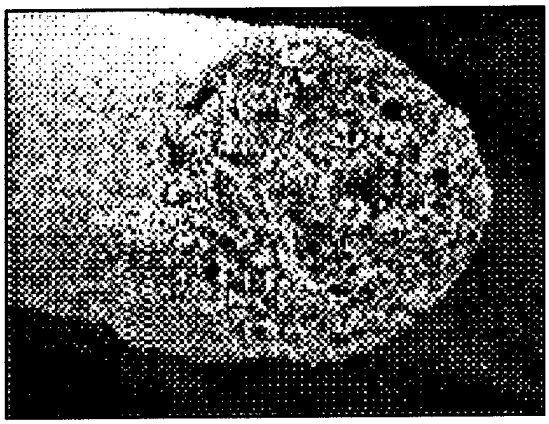


Figure 4. Scanning Electron Microscopy of an extruded AG in tubular form.

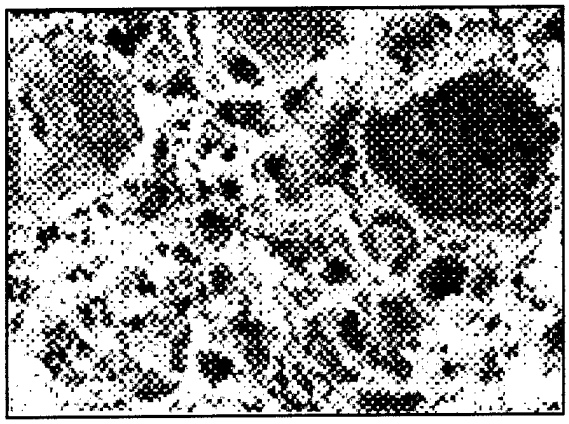


Figure 5. Scanning Electron Microscopy in cross section at higher magnification of AG

Vascular Endothelium Growth Factor (VEGF) is produced by many different cell types both in tissue culture and in vivo. It binds to plasma membrane receptors on endothelial cells (EC) only with an extra cellular transmembrane glycoprotein linked to an intracellular tyrosine kinase domain, thus VEGF is an EC specific mitogen.

VEGF-C is a 38 kD homodimeric glycoprotein that is commercially available. This recombinant human VEGF-C is from a DNA sequence encoding the 165 amino acid residue variant of human VEGF expressed in Sf 21 insect cells using a baculovirus expression system. The product is lipolized from a sterile-filtered solution in 30% acotonitrile plus 0.1% TFA containing 50μg of cytokine. **VEGF-C** was of particular interest to us because it is a specific mitogen for **lymphatic endothelium** in adult tissues.

For the In Vitro studies we were going to conduct we chose VEGF-C as our growth factor to incorporate into the alginate gels for its slow sustained release, in an attempt to promote lymphatic endothelial cell proliferation and migration.

In order to asses the ideal dose of VEGF-C incorporated into the alginate gels we proceeded to isolate lymphatic endothelial cells from rat thoracic ducts to have an available cell line for further *in vitro* studies.

Animal model for LEC harvest:

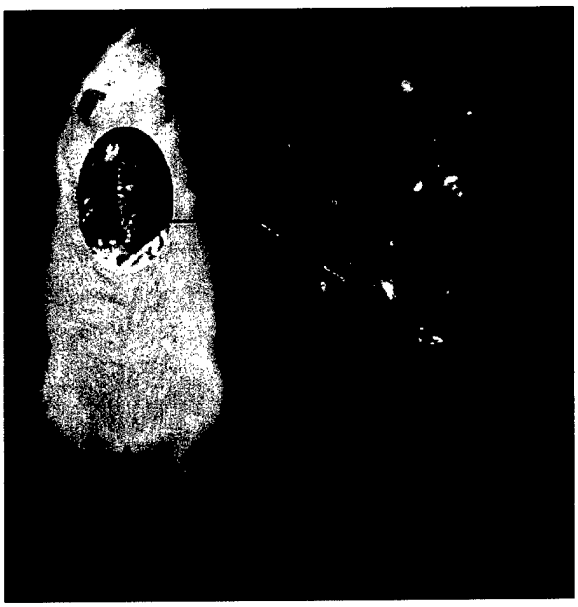


Figure 6. Photograph of Animal Model for LEC Harvest. Thoracic Aorta (THA), Thoracic Duct (THD) and Lymphatic Endothelial Cells (LEC)

Six Wistar rats (250-300g) were used for Thoracic Duct dissection and harvest. Animals were anesthetized by intraperitoneal injection of pentobarbital (40-50mg/Kg). Once the abdomen of the animals was prepared for surgery, a longitudinal incision was made to enter the abdominal cavity. The abdominal viscera were then rejected to the side and dissection from the cysterna chyli to the thoracic duct was performed. Once the structure was isolated it was cutout and placed in a sterile tube w Endothelial Cell Media for preservation and placed on ice. The animal was then euthanized by an overdose of the anesthetic (Animal procedures complied with the "Principles of Laboratory Animal Care" NIH No. 80-23, revised 1978).

Thoracic ducts (TD) were then collected from the sterile tubes with EC media on ice to retrieve the LEC's following a sterile procedure at all times.
Under a laminar flow hood the TD's were taken out, placed in a petri dish and submerged in M199. At one end a 30g canula was inserted and tied. At the other end a mini vascular clip was placed on. The TD was then infused with a 0.1% collagenase solution. A second mini vascular clip was placed on at the site of infusion so that the TD's would be distended w the collagenase solution. The TD's were left on the petridish submerged under M199 for 20 minutes at 37°.

In a 15 ml falcon tube 5 ml of M199 media were added, then, after 20 minutes, the TD's distal mini vascular clip was removed making sure that all the collagenase solution from within would go into the falcon tube.[23]
The 15 ml falcon tube was then centrifuged at 1 200 rpm's for 5 minutes. The media was aspirated and cells were resuspended in 2 ml of EC media. Approximately 8 ml of EC media were poured into a gelatin coated plate and then the 2 ml of EC media with the LEC that were resuspended, transferred to the plate. EC media was changed every 2 days until cells reached confluence. They were tripsinized, removed form the plate to be frozen (-80°) in 0.5 ml of EC media for later use.

Preliminary in vitro studies were carried out to determine the ideal VEGF-C concentration to be incorporated into the alginate gels. In vitro culture with LEC and three different concentrations, 50, 100 and 200 ng/ml of VEGF-C reveled that the proliferative and migratory response of the LEC was greater at 200 ng/ml when compared to the other two concentrations.

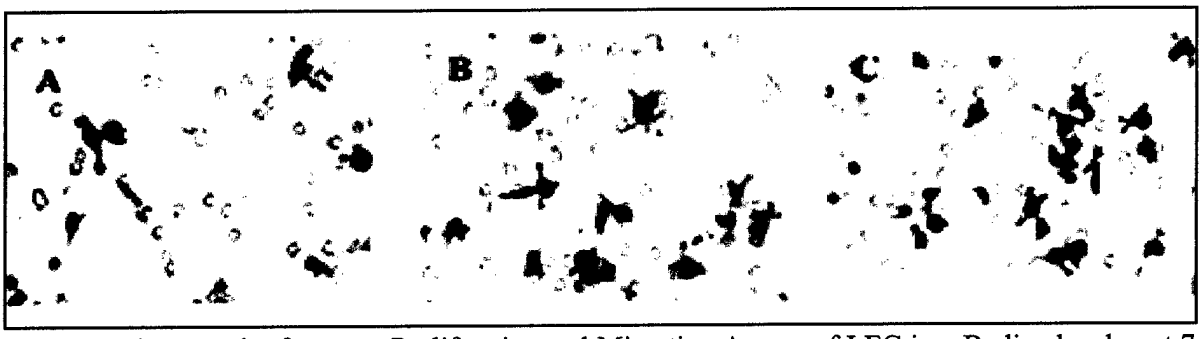


Figure 7. Photograph of *in vitro* Proliferation and Migration Assays of LEC in a Bodin chamber at 7 days. A) VEGF-C concentration of 50 ng/ml. B) VEGF-C concentration of 100ng/ml and C) VEGF-C concentration of 200 ng/ml.

With these initial results we determined that the concentrations of VEGF-C that should be incorporated into the alginate gels should be the same as the ones used above, and determine if the release of the growth factor from the gel would result in the same or comparable proliferation and migration of LEC (Figure 8).
LEC were cultured with Ham's modified F12K medium for EC with ECGS. Cells were serum starved (from 10% to 1% FBS) prior to plating them on the petri-dish with the alginate gels.

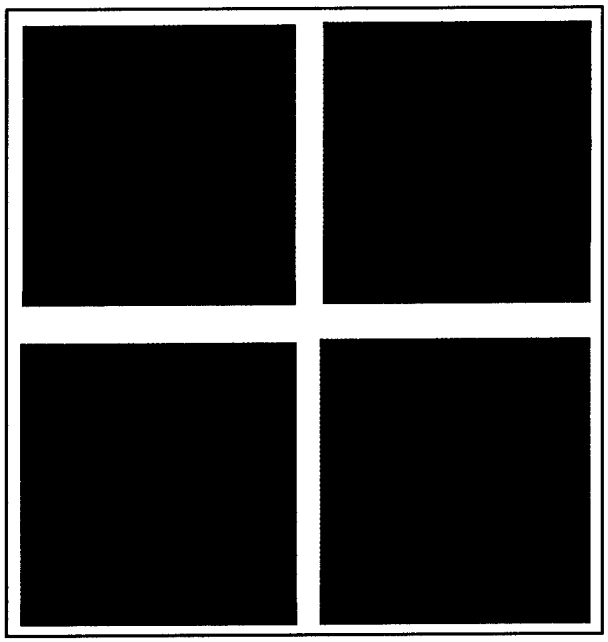


Figure 8. Photograph of *in vitro* proliferation and migration of LEC.

A) Plain Alginate Gel, no migration or proliferation of LEC. B) There is evidence of proliferation and migration of LEC towards the VEGF-C (50ng/ml) Alginate-Gel, however, more pronounced on picture C, wherethe VEGF-C concentration was higher (100ng/ml). D) Evidence of proliferation and migration of LEC with tubular formation along the VEGF-C (200 ng/ml) Alginate-Gel.

KEY RESEARCH ACCOMPLISHMENTS:

Our Specific aims for the first year of these award were successfully completed.

* We were able to produce a porous, biodegradable gel with alginate to use as a delivery device for VEGF-C growth factor to promote Lymphatic Endothelial Cell proliferation and migration *in vitro.*

* Next phase we'll incorporate Angiopoietin-1 to the VEGF-C alginate gels to enhance the proliferative and migratory response of the LEC.

REPORTABLE OUTCOMES:

* Rat Thoracic Duct Endothelial Cell line was created.

* Abstract submitted to the International Meeting on Angiogenesis & Lymphangiogenesis: Basic Mechanisms and Therapeutic Implications was accepted for Poster presentation, and was printed in the Meeting's Publication.

* We were invited to present our work as an Oral presentation at the Era of Hope, September 2002 meeting.

* We were invited by Dr. Stanley G. Rokson, Chief editor of the *Lymphatic Research and Biology Journal* to submit a manuscript this fall. Manuscript in progress.

CONCLUSIONS:

A biodegradable hydrogel such as Alginate, may be a good delivery system for sustained slow release of VEGF-C to promote lymphangiogenesis in those instances where the integrity and function of the VEGF-3 receptor of LEC is intact. Further studies in lymphedema animal models are required to evaluate *in vivo* this methodology.

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APPENDICES:

- Copy of Poster & Publication presented at the International Meeting on Angiogenesis Basic Mechanisms and Therapeutic Implications.

LYMPHANGIOGENESIS FROM POROUS ALGINATE-VEGF-C-HYDROGELS *IN VITRO*

Mauricio A. Contreras, MD; Evan R. Deutsch, MD; Matthew D. Phaneuf, BS and Sumner A. Slavin, MD
Beth Israel Deacones Medical Center, Division of Plastic Surgery, Harvard Medical School

ABSTRACT

Introduction: The lymphatic vascular system develops from embryonic veins through a process referred to as Lymphangiogenesis. This event occurs in parallel with maturation of the blood vascular system,aberrant lymphangiogenesis contributes to interstitial protein accumulation, leading to a continual increase of osmotic pressure and thus fluid accumulation. Lymphedema therefore, ensues as a result of lymphatic blockage, trauma or dysfunction. Objective: To promote Lymphangiogenesis by stimulating lymphatic endothelial cell (LEC) proliferation and migration using an Alginate-VEGF-C hydrogel *in vitro*. Methods: Sterile powder alginate (G/M composition) was mixed with Potassium Phosphate and Sodium Chloride (0.1 M K_2HPO_2 and 0.135 M NaCl, ph 7.4) previously sterilized by autoclave. This solution was then added to sterile powder VEGF-C at three different concentrations 50, 100 and 200 ng/ml. The alginate-VEGF-C mixture was then extruded through a 25g needle and solidified under sterile conditions by internal geleation method/internal setting (Ca^{2+}). Previously harvested and cultured Lymphatic Endothelial Cells from rat thoracic duct were placed on a petri dish and cultured (EC media) with the various alginate-VEGF-C hydrogels and a plane alginate hydrogel as control. Results: The LEC migrated towards all of the alginate-VEGF-C hydrogels and proliferated along their length. However, the higher concentration of 200 ng/ml of VEGF-C exhibited a more profound effect on migration as well as proliferation, forming tubular structures. The plane alginate hydrogel had no effect on LEC migration or proliferation and there were no tubularstructures formed. Conclusions: The results of this *in-vitro* study indicate that an alginate biodegradable hydrogel may be an effective delivery system for VEGF-C. Its potent mitogenic effect on LEC could play an important role in Lymphangiogenesis. Further in vivo animal studies are required to evaluate alginate-VEGF-C hydro- gels and their potential role in restoring the lymphatic circulation in Lymphedema.

INTRODUCTION

Hydrogels have been increasingly important in biotechnology applications, such as tissue engineering, artificial organs and drug carriers, specially for proteins.[1] Delivery rate can be predetermined and its local, rather than, systemic administration be of great advantage.

VEGF is a secreted EC mitogen with high affinity binding sites limited to EC's.[2] VEGF-C was discovered as the ligand for the receptor VEGF-3, which is essential for embryonic cardiovascular development, but thereafter becomes confined to the lymphatic endothelium in adult tissues.[3]

METHODS

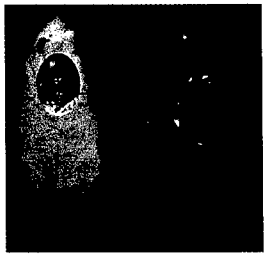

Figure 1: Thoracic Duct Lymphatic Endothelial Cell Harvest.

RESULTS

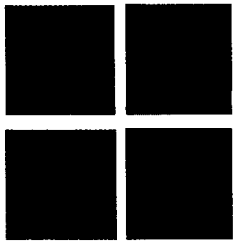

Figure 2: A) Plain Alginate Gel, no migration or proliferation of LEC. B) There is evidence of proliferation and migration of LEC towards the VEGF-C (50ng/ml) Alginate-Gel, however, more pronounced on picture C, where the VEGF-C concentration was higher (100ng/ml). D) Evidence of proliferation and migration of LEC with tubular formation along the VEGF-C (200 ng/ml) Alginate-Gel.

LEC were harvested by infussion of 0.1 collagenase solution [4] (1% FBS, 1% P/S, M199 and filtered) of the thoracic duct. LEC were cultured with Ham's modified F12K medium for EC with ECGS. Cells were serum starved (from 10% to 1% FBS) prior to plating them on the petri-dish with the alginate gels.

CONCLUSIONS

A biodegradable hydrogel such as Alginate, may be a good delivery system for sustained slow release of VEGF-C to promote lymphangiogenesis in those instances where the integrity and function of the VEGF-3 receptor is intact. Further studies in lymphedema animal models are required to evaluate *in vivo* this methodology.

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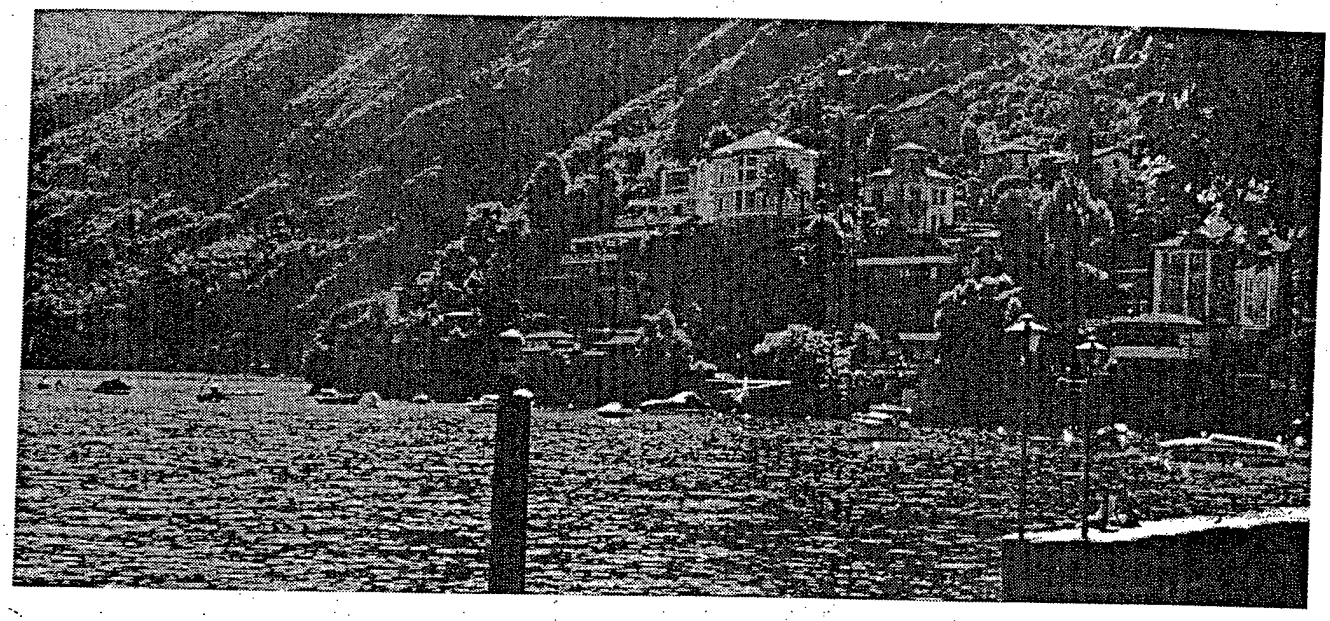
ANGIOGENESIS: BASIC MECHANISMS AND THERAPEUTIC IMPLICATIONS



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LYMPHANGIOGENESIS FROM POROUS ALGINATE-VEGF-C HYDROGELS IN VITRO

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Introduction: The lymphatic vascular system develops from embryonic veins through a process referred to as Lymphangiogenesis. This event occurs in parallel with maturation of the blood vascular system, aberrant lymphangiogenesis contributes to interstitial protein accumulation, leading to a continual increase of osmotic pressure and thus fluid accumulation. Lymphedema therefore, ensues as a result of lymphatic blockage, trauma or dysfunction. Objective: To promote Lymphangiogenesis by stimulating lymphatic endothelial cell (LEC) proliferation and migration using an Alginate-VEGF-C hydrogel in vitro. Methods: Sterile powder alginate (G/M composition) was mixed with Potassium Phosphate and Sodium Chloride (0.1 M K2HPO2 and 0.135 M NaCl, ph 7.4) previously sterilized by autoclave. This solution was then added to sterile powder VEGF-C at three different concentrations 50, 100 and 200 ng/ml. The alginate-VEGF-C mixture was then extruded through a 25g needle and solidified under sterile conditions by internal geleation method/internal setting (Ca2+). Previously harvested and cultured Lymphatic Endothelial Cells from rat thoracic duct were placed on a petri dish and cultured (EC media) with the various alginate-VEGF-C hydrogels and a plane alginate hydrogel as control. Results: The LEC migrated towards all of the alginate-VEGF-C hydrogels and proliferated along their length forming tubular structures, however, the higher concentration of 200 ng/ml of VEGF-C exhibited a more profound effect on migration as well as proliferation. The plane alginate hydrogel had no effect on LEC migration or proliferation and there were no tubular structures formed. Conclusions: The results of this in vitro study indicate that an alginate biodegradable hydrogel may be an effective delivery system for VEGF-C. Its potent mitogenic effect on LEC could play an important role in Lymphangiogenesis. Further in vivo animal studies are required to evaluate alginate-VEGF-C hydrogels and their potential role in restoring the lymphatic circulation in Lymphedema.